

**Report for 2004MT34B: Investigation of microbial ecology, structure, and function in coalbed aquifers: Powder River Basin, Montana**

There are no reported publications resulting from this project.

Report Follows

## **Investigation of microbial ecology, structure, and function in coalbed aquifers: Powder River Basin, Montana**

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**Current Status:** Preparing final report

Coalbeds supply three critical resources in southeastern Montana: 1) coal for energy; 2) water for domestic and agricultural uses; and 3) coalbed methane. Currently, these aquifers are being impacted by conventional coalbed methane (CBM) development. As concerns of global warming increase, speculation that these aquifers may serve as repositories for industrial CO<sub>2</sub> suggests that additional impacts are likely in the future. A comprehensive understanding of the relationships between the hydrogeologic terrains and the total microbial community at depth will help establish best management practices for methane production and potential CO<sub>2</sub> sequestration. Avoiding waste of the methane resource and the associated produced water is key to comprehensive resource sustainability that will preserve the aquifer yet allow for long-term recovery and utilization of methane and may help remediate the atmospheric CO<sub>2</sub>.

Methane is held on cleat faces and micropore surfaces in coal by a combination of physical sorption and hydrostatic pressure from ground water in the coal (Law and Rice, 1993; Rightmire, and others, 1984), and is released when the water pressure is reduced. To reduce hydrostatic pressure and capture released gas, water is pumped from wells drilled and completed in coalbeds.

The origin of CBM in the Powder River Basin (PRB) is the result of microbial processes (biogenic methanogenesis). The success of CO<sub>2</sub> sequestration strategies will likely be a function of microbial activities as well. The purpose of this research is to begin the process of identifying the structure, diversity and presumptive function of the total microbial community and ecology within a specific methane-bearing coalbed aquifer in the PRB and conduct culture-based investigations that will help delineate the kinetic rates and pathways for methanogenesis. We foresee the value of data collected during this project as a means of moving us toward a philosophy of harvesting CBM rather than simply mining this resource at the expense of ground-water resources.

There are two distinct types of ground-water flow systems in the Powder River Basin, a deep regional system and a series of local flow systems. Ground water flows generally from the south to the north, with flow in the local systems reflecting topographic control. Recharge occurs at outcrop areas around the edges of the Basin in Wyoming and in high clinker-capped ridges such as the Wolf Mountains in Montana (Wheaton and Donato, 2004). Coal seams are the most continuous water-bearing geologic units and have hydraulic conductivity values equal to or slightly greater than those in sandstone aquifers.

Due to the geologic structure of the Powder River Basin, and the topographic relationship between generally higher elevations in Wyoming and lower elevations in Montana, coal seams outcrop along valley walls in Montana and ground-water discharges as springs at these outcrop areas. Additional ground-water discharge occurs as baseflow to streams and rivers in Montana.

The quality of ground water in the Powder River Basin reflects chemical and biological reactions that occur along flow paths. In deep coal beds, such as those that contain coalbed methane, chemical reactions have greatly reduced the amounts of sulfate, calcium, and magnesium, so that the water quality is dominated by moderate concentrations of sodium and bicarbonate. Coalbed methane can only exist in the sulfate-depleted, anaerobic conditions which occur in deeper coals. Therefore, all CBM production water is rich in sodium and much of it has a high SAR value.

It is understood that biogenic methane is produced as an end-product of a complex set of metabolic pathways represented by a consortium of microorganisms, including members of the domains *Eubacteria* and *Archaea*. This intricate and closely associated assemblage resides in anoxic zones depleted of typical electron acceptors found in many subsurface environments. Four groups of functionally diverse prokaryotes have been identified, as being necessary for the formation of methane under these conditions: 1) hydrolytic bacteria, 2) fermentative bacteria, 3) acetogenic bacteria and 4) methanogens (Whiticar, 1999). Each of these guilds of microorganisms is responsible for an important function in the methanogenic pathway. The hydrolysis of higher molecular weight substrates, such as, cellulose, high molecular weight proteins and mixed composition polysaccharides by hydrolytic and cellulolytic competent bacteria is a necessary first step in the decomposition of organic materials. It has been postulated that this represents the rate limiting step in the formation of methane in anoxic environments. Following their breakdown into monomeric subunits such as short-chain fatty acids, sugars, amino acids

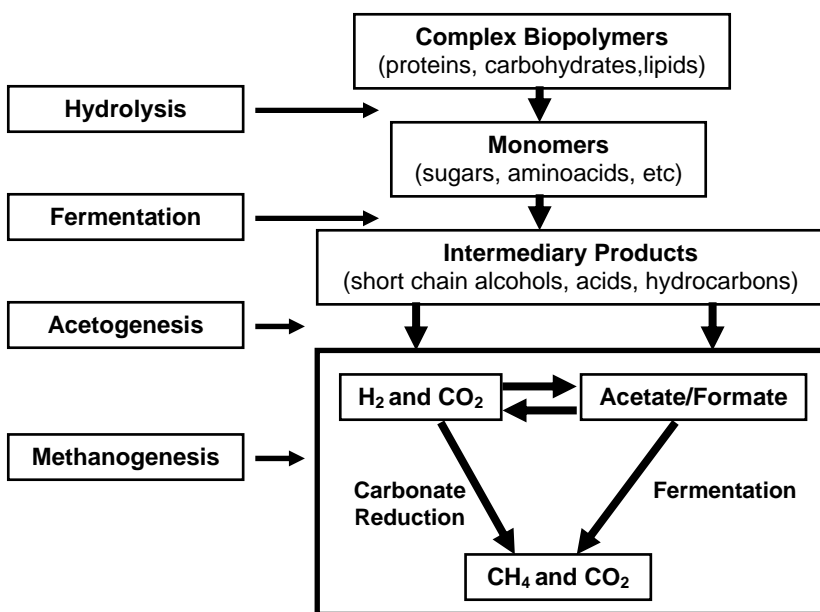
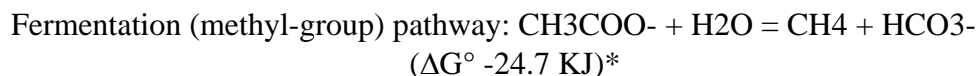
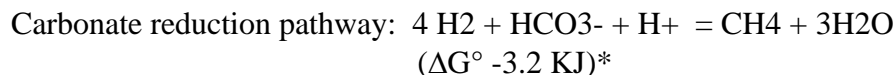


Figure 1. Anaerobic Degradation of organic compounds

and additional substrates (e.g. ammonia and hydrogen sulfide), fermentation proceeds, mediated by assorted fermentative bacteria. The fermentation process produces a number of byproducts, including additional short-chain alcohols and acids (propionate and butyrate are common), as well as, acetate, formate and carbon dioxide

(CO<sub>2</sub>). Because methanogens metabolize a narrow range of compounds and are restricted to anoxic environments with redox potentials of  $E_h < -200$  mV (Budwill, 2003), some further degradation is assumed to be required. Syntrophic acetogenic bacteria play an important role in consuming many of the short-chain acids that accumulate in the pathway, and the end products, predominately acetate and CO<sub>2</sub>, become viable substrates for methanogenesis. The final step prior to conversion to methane is to convert any remaining alcohols and acids into acetate, carbon dioxide (methanol and methylamines may also be substrates for methanogenesis), hydrogen (H<sub>2</sub>) and in some cases formate. This general scheme of anaerobic degradation of organic compounds to methane is diagrammed in figure 1.

Although the reduction of carbon dioxide by hydrogen is thought to be the most commonly used method for the production of methane in anoxic environments (Scott, 1999), the reduction of acetate (or a very limited methyl group containing hydrocarbon) provides a greater change in free energy and therefore is more favorable for energy conservation. This situation remains unclear, as the opposite is thought to be true in certain environments such as marine or open freshwater settings (Whiticarr, 1986). The two pathways may operate simultaneously under some circumstances and at differing stages of sedimentation of organic materials (Kotelnikova, 2002). While each of the two reductive processes produces methane, the two separate pathways may be active. The former by the (hydrogen mediated reduction of carbon dioxide) carbonate reduction pathway and the latter by the fermentation pathway. The general chemical equations for the respective pathways are illustrated below.



\*Reported free energy values vary from source to source.

Evidence supporting the notion that the two pathways operate at various times was reported by Chin et al, (2003). They described temporal changes in methanogen production in flooded rice paddies. Their findings indicate that structural changes in the methanogenic community lead to functional changes in methane production with time. Similarly Scheid et al. (2003) using rice roots as a community model for methanogenesis were able to show methanogenic community shifts when nitrate and sulfate were introduced. Apparently, the addition of alternative electron acceptors leads to changes in community substrate usage and may have broad effects on community structure and activity. Likely, competition between methanogens and sulfate and nitrate reducing bacteria led to these changes.

The use of culture independent molecular techniques for our investigation is crucial. It is generally accepted that classic culturing techniques may under represent microbial diversity in typical environments by two to three orders of magnitude (Torsvik, 1994; Torsvik et al., 1990). It is apparent that microbial communities and their associated

populations play important roles in biogeo-chemical and physicochemical processes including methanogenesis and carbon cycling. Functional guilds of bacteria that have been associated with biogenic methane production include hydrolytic and cellulolytic bacteria, fermentative and acetogenic bacteria, as well as methanogens (Whiticar, 1999). However; Polman et al. (1993) reported that there were no viable microorganisms in three different ranked coals. Their observations were based on results of experiments attempting to grow bacteria in cultures. Vorres, (1990) reported that anaerobically preserved Argonne Premium Coals produced methane in sealed ampoules. Also these samples contained cultivable *Clostridium* species. In 1994, work by both Johnson et al. and Volkwein et al. noted that higher-rank coals produced low molecular weight organic acids when they were inoculated with presumptive anaerobic consortiums from various sources. Based on additional work completed by these groups, they concluded that the microorganisms collected from those environments (that were likely to contain methanogens and other consortium members) were responsible for the production of the methane. In Volkwein et al. (1994), although their cultures remained viable and continued to produce methane through five successive transfers, they were unsuccessful at identifying any of the microorganisms.

The purpose of the proposed research is to elucidate the diversity, composition, activity and function of the methane producing microbial community in coalbeds. These findings will have broader impacts than simply exploring the microbial ecology of a novel subsurface environment. Understanding the nature of the microbial ecology of coalbed seams will contribute knowledge toward management of enhanced microbial methane production and recovery and possibly contribute to CO<sub>2</sub> sequestration efforts, thereby impacting greenhouse gas mitigation strategies.

### **Sample collection**

Two microbial samples were collected in Wyoming from the Tongue River Member, Big George coal seam. A water quality sample was collected at a nearby CBM discharge point from wells completed in the same coal seam, but different from the well where the coal samples were collected. The sites are in the Powder River watershed in east central Johnson county. The coal samples were collected during under reaming, using forward air rotary, of an already cased CBM well. The samples were gathered from the diverter pipe on the drill rig with a sample screen in less than one minute the samples were inserted into an anaerobic chamber with an oxygen consuming package then sealed. The coal samples were held in cold storage until they arrived at the laboratory at the University of Montana. The water sample was submitted to the Montana Bureau of Mines and Geology analytical laboratory for analysis.

The upper coal sample was collected while reaming from 1525 feet to 1530 feet. The second coal sample was collected just after reaching the base of coal (1596 feet) while cleaning the borehole. Since the well was cased, neither coal sample contained material from farther up the bore hole, and appeared clean and in good condition.

Analytical results of the water quality sample indicated total dissolved solids concentration of 2,056 mg/L, SAR of 25.5 and the sulfate concentration of less than 2.5 mg/L. The water quality is typical of all CBM production water in the PRB.

## **Molecular Analysis:**

### **Nucleic Acid extraction**

**Aqueous Phase:** To increase the biomass for molecular analysis, cells were collected by filtration onto three separate 142-mm Supor (Pall Corporation, Ann Arbor, MI) 0.2 µm membrane filters. Each filter received an approximate equal volume of groundwater (approximately 13 liters). Filters were placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI) and frozen at -80° C. Prior to genomic DNA extraction, the frozen filter was crushed thoroughly within the collection bag. Processing of total community DNA from the filter was carried out by the direct lysis method of Holben (1997) with minor modifications.

Briefly, 20 ml of autoclaved extraction buffer (200 mM sodium phosphate buffer (NaPO<sub>4</sub>), 100 mM ethylenediamine tetra-acetate (EDTA) and 1.5% sodium dodecyl sulfate (SDS), pH 8.0) was added to sterile Oak Ridge tubes containing sterile glass beads (5 g of 0.2 mm and 5 g of 1 mm diameter) (Sigma Chemical Co., St. Louis, MO.). To this tube, one macerated filter was added, placed in a 70° C water bath for 30 min with frequent vortexing (5 min intervals). Tubes were then placed on a reciprocal platform shaker and shaken on high (approximately 100 oscillations/minute) for 30 min at room temperature. Filter, particulate and cell debris were removed by centrifugation (Sovall RC 5B Plus with SS34 rotor) at 10,000 RPM (7,796 x g) for 10 min at 10° C. Supernatant was transferred to clean Oak Ridge tubes and incubated on ice for 30 min to precipitate the SDS, then centrifuged as above to pellet SDS. Liquid was transferred to new 50 ml tubes with addition of 10% volume 3 M sodium acetate (pH 5.2) and 2.5 volumes 100% cold ethanol. After overnight incubation at -20° C, nucleic acids were collected by centrifugation, as described above. Nucleic acid pellets were resuspended in approximately 1 ml of sterile deionized water and precipitated by addition of 2.5 volumes 100% cold ethanol and placed in at -20° overnight. After centrifugation (as above) the resulting nucleic acid pellet was air dried and suspended in approximately 500 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

**Solid Phase:** Anaerobic coal samples were subjected to direct nucleic acid extraction, as well as, used as inocula in both groundwater and growth media. Direct nucleic acid extractions were performed using Power Soil DNA Extraction Kits (Mo Bio, Solano Beach, CA) as per manufacture's suggestion. In addition to standard extractions, coal samples were subjected to further DNA purification which included the addition of chaotropic salts and silicon binding matrices. This method has proven to be beneficial when attempting to amplify various environmental samples.

Resulting DNA from both solid and aqueous phases was subsequently subjected to DNA amplification by the Polymerase Chain Reaction (PCR) using both generally conserved primers and methanogen specific primer sets 16S rDNA primers (536fc and 907r) as well as the methanogen-specific primer pair 23fc and 440r). More recently, the genomic DNA was amplified with primer set ME1 and ME2, which are specific for the *mcr* (methyl coenzyme M reductase) gene (alpha subunit). The expected product is approximately 750 kb. The result of this gave correct size product, which were gel purified and are to be used to align with groups of other amplicons derived from Powder River Basin coal

Figure 2. DGGE analysis of the microbial community of PRB coal associated aquifer water.

within the order *Methanosarcinales*. In addition, all five were most closely related to the species *M. taylorii* or *M. oregonensis* (averaging 93% homology). Interestingly and unexpectedly, this group is typically linked with marine environments. However, the geological formation and shallow depth where this sample was taken have not been associated with ancient marine origins. This evidence supports the concept that this environment may sustain novel members of the methanogen group. More recently, an additional survey of coal methanogens (using ME1 and ME2) produced seven sequences all related to members of the *Methanosarcinales* or *Methanobacteriales* orders. Six appear to be unique from each other and their closest matches are to environmental clones from various origins. Additionally, many of the important members of the consortia may be underrepresented in terms of numbers, but may be dominant in terms of activity. If this were the case, it suggests that there are a number of minority microbial populations are present in coalbeds, and that to more fully understand the community ecology an extensive and intensive investigation must be undertaken.

In addition to the molecular data we are currently in the midst of incubating coal samples in our laboratory. As of yet there is little evidence of growth, based on turbidity and direct observations. However this is not surprising as methanogen growth is typically very slow and may indeed take a period of time well beyond the time frame of this study. However these culture attempts will continue and a molecular analysis of these samples will be undertaken.

### **Conclusions and recommendations:**

This initial investigation proved to be an excellent starting point for continuing efforts toward unraveling the microbial community complexity responsible for biogenic methane production. To fully underpin the microbial community in this environment a more comprehensive study must be undertaken which would include the following:

- Continue with sequencing efforts on the microbial community within coal samples
- Develop conceptual model of the microbial community present based on molecular analysis
- Design primers appropriate for real-time PCR
- Conduct culturing experiments and isolate pure cultures to confirm the presence of novel organisms
- Design amendment/perturbation experiments for laboratory (later for *in situ*?)
- Develop activity studies aimed at determining active microbial populations responsible for methane production (future stable isotope experiment)